Virtual Pinhole Confocal Microscope

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Introduction

For 400 years optical microscopy has been the principal method for the study of biological structure at the cellular level. Over the past century, the application of photography has allowed the capture and distribution of microscopic images to a wide audience. Optical microscopy, considered a mature technology for years, has undergone a renaissance in the past two decades. The advent of electronic imaging is producing a revolution in scientific microscopy that is redefining the frontiers of speed, temporal and spatial resolution, sensitivity, and perhaps most importantly, the capacity for quantitative photometric, spectral, and geometric measurements. New optical and imaging strategies are disclosing new structural and dynamic functional details of the microscopic domain. Confocal imaging techniques have provided striking improvements in the spatial resolution of optical microscopy, providing true three-dimensional (3-D) volume imaging.

Confocal Principle and Practical Instruments

The key insight that has driven the development of confocal imaging, is summarized by Marvin Minsky, a noted pioneer in the development of artificial intelligence, and in 1955 the inventor of a confocal microscope:

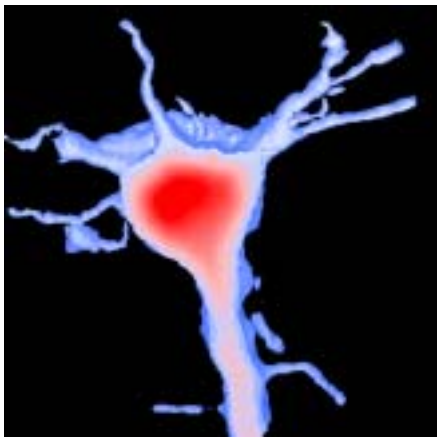


Figure 1. A three-dimensional (3-D) rendering of a nerve cell reconstructed from a series of virtual pinhole microscope (VPM) confocal images.

"An ideal microscope would examine each point of the specimen and measure the amount of light scattered or absorbed by that point. But if we try to make many such measurements at the same time, then every focal image point will be clouded by aberrant rays of scattered light that are deflected from points in the specimen that are not the points you are looking at.... It is easy to remove all rays not initially aimed at the focal point; just use a second microscope (instead of a condenser) to image a pinhole aperture on a single point of the specimen.... Still, some of the initially focussed light will be scattered by out-of-focus specimen points onto other points in the image plane. But we can reject those rays as well, by placing a second pinhole aperture in the image plane that lies behind the exit side of the objective lens. We end up with an elegant, symmetrical geometry: a pinhole and an objective lens on each side of a specimen."

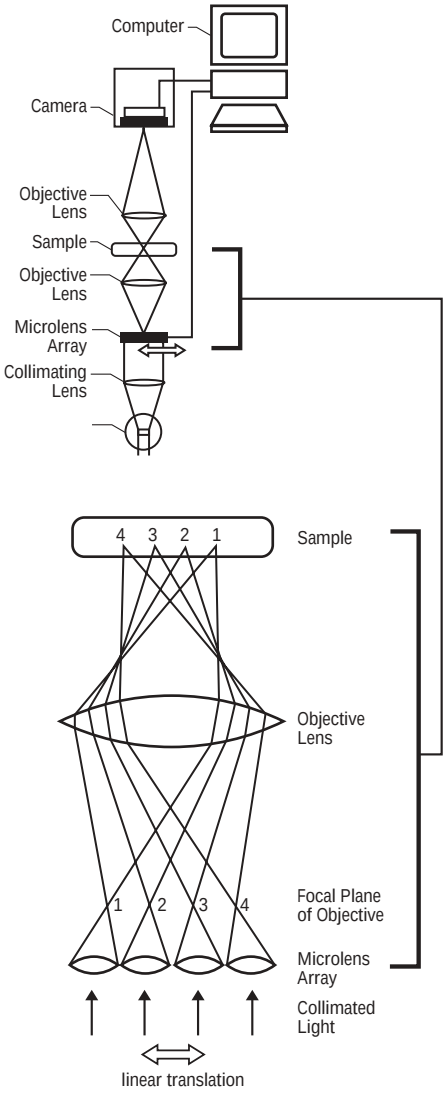


Figure2. Conventional transmission confocal microscope. The most practical implementations scan the specimen stage to maintain optical alignment in the illumination and imaging path.

A conceptually simple embodiment of a confocal microscope of the sort described by Minsky is illustrated schematically in Figure 2. The system is configured for transmission imaging. Light from a source is transmitted through a pinhole. Light from the pinhole is brought to a point focus within the sample volume by imaging optics. Light emerging from the sample is brought to focus on a pinhole aperture by a second set of imaging optics. This aperture acts as a spatial filter for imaging, rejecting most of the light that did not pass through the confocal point in the sample volume, thereby reducing image contributions from light scattered or refracted from other locations within the medium. The detector for such a system typically consists of a single-channel device such as a photodiode or photomultiplier.

In order to collect an image, it is necessary to scan the illumination and detection subsystems relative to the sample. However, the technical requirements for coupled electromechanical scanning to preserve the required optical alignment between illumination and imaging pathways are formidable. Most practical embodiments of a transmission confocal system utilize mechanical scanning of the sample so that the imaging elements do not move, however scanning in such systems is very slow.

Confocal microscopy systems offer a number of advantages for quantitative imaging including improved image contrast, resolution, and limited depth-of-field. The limited depth-of-field of scanned confocal microscopes has supported applications not previously feasible, such as the optical sectioning and volumetric reconstruction of complex subcellular structures, or mapping the spatial and temporal distributions of intracellular ions. Conventional confocal systems incorporate several key functional subsystems:

- a system for scanned illumination;
- a spatial filter or aperture in the focal plane of the detector to reject out-of-focus light; and
- a single channel or imaging detector.

Imaging optics bring the illumination and imaging paths into focus at the same location within a sample—hence the term “confocal”.

Most existing commercial systems are designed for epifluorescence imaging (*i.e.*, using reflected light), allowing scanning and aperture functions for illumination and imaging to be performed by a single piece of hardware. Initial commercial confocal microscopes appearing in the mid-1980s utilized laser illumination and single-channel photomultiplier detectors. Although a number of important technical advances have emerged over the intervening years, most confocal systems remain complex, inflexible, slow, and expensive.

At Los Alamos National Laboratory, we have developed a novel approach for confocal microscopy that uses available illumination, detection, and data-processing technologies to produce an imager with a number of advantages: reduced cost, faster imaging, improved efficiency and sensitivity, improved reliability, and much greater flexibility. This system has three key components:

1. an electronically or electromechanically scanned illumination subsystem,
2. an area sensor such as a charge-coupled device (CCD) imager, and
3. a "virtual pinhole"—synthetic aperture constituted during image processing after sensor readout.

Our approach provides the core technologies for a family of microscopic-imaging methods that will significantly advance the utility of optical microscopy for clinical applications and basic research. The initial focus of our work has been the development of a low-cost system for confocal imaging suitable for retrofit integration with conventional research microscopes. However, our approach also offers simple and effective solutions for transmission confocal imaging, a practical approach for confocal imaging through an endoscope, and for high-performance confocal spectral imaging.

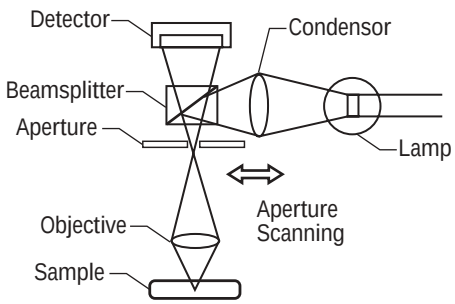


Figure3. A conventional confocal microscope configured for epifluorescence or reflected-light imaging. The aperture configuration and scanning strategies vary widely.

Confocal Imaging Approaches

The conventional configuration for confocal imaging in reflected light or epifluorescence modes is illustrated in Figure 3. This configuration employs the same imaging optics and often the same pinhole aperture for both the illumination and imaging paths. This arrangement simplifies optical alignment and facilitates optical scanning by mechanical movement of the aperture, or by shifting the image of the aperture by mirrors in the optical path. In many designs the scanning of the illumination path and the "descanning" of the imaging path are accomplished with the same set of mirrors. Image acquisition in most existing systems is relatively slow, which complicates both the preliminary inspection of a sample and focusing.

Spinning-disk confocal systems are a useful implementation of the principles illustrated in Figure 3. In such systems the scanning and aperture functions are embodied in an opaque disk containing a pattern of optically transmissive pinholes. These systems can significantly enhance the speed, flexibility, and ease of use of confocal microscopy. However, they typically suffer from low sensitivity (illumination is inefficient because most light is absorbed by the opaque regions of the disk) and from reflection from the disk surface. Another strategy for high-performance imaging is to use an illumination source configured as a line instead of as a point, with a slit aperture instead of a pinhole. In a line-scan configuration, out-of-focus light that happens to fall onto the slit aperture contributes to the image, however in practice the degradation of confocal image quality is not unacceptably severe.

An alternative approach, developed by Greenberg and colleagues at NIH, in principle offers higher performance because the need for mechanical scanning is eliminated. The system scans a laser in two dimensions using a pair of optoacoustic beam-steering devices. The detector is based on an image dissector videcon—a television detector tube that allows random access readout of the tube photocathode at user-defined coordinates (defined by a pair of voltages). However, like most other tube-based video detectors, the image dissector has been rendered obsolete by solid-state video devices, and a commercial version of this confocal imager has not been produced.

Virtual Pinhole Microscope—A New Approach

Our virtual pinhole microscope (VPM) can use a method analogous to slit scanning for fast, full-field scanning. Because the system integrates image-processing techniques, it can correct image degradation associated with slit scanning. The video-scan configuration of the VPM allows fast, continuous confocal point measurements at arbitrary locations within a sample. Even using standard video technologies, the VPM provides full-frame imaging rates comparable to those of most laser-scan confocal instruments. The VPM can reconstruct and display clear point-scan confocal images in real time using high-performance video cameras and a data-acquisition system that we developed. The low-cost, high-speed CCDs that are now available can achieve full-field imaging rates approaching 1,000 frames per second (or more, with slightly more complex devices). Though typically lower resolution than standard video cameras, these CCDs are particularly well-suited for "hyperacuity" imaging: a reconstructed VPM image can have higher resolution than the camera used to collect the component images.

Our approach to confocal imaging operates on the same principles as existing confocal imagers, but realizes the necessary functional subsystems in a novel way. The first key subsystem is a system for electro-optically scanned illumination. Several classes of technology satisfy our basic requirements for speed and precision. Scanned illumination is achieved by the use of spatial light modulators (SLMs)—devices that can be electronically driven to produce time-varying spatial patterns of illumination.

We have explored the use of display devices used in video projectors. Most computer-compatible video projectors now employ liquid crystal devices (LCDs). Texas Instruments recently introduced digital light processor (DLP) technology, based on an electronically addressable micromirror array. In our usual scanning method, only a small fraction of the total number of pixels is illuminated at any given time, typically on the order of 1 in 25. Separation between illuminated spots is maintained to avoid cross-talk between scanned points. However, most (e.g., 96%) of the available light is wasted. For some applications this poses little concern and the flexibility of programmable illumination and solid-state devices justifies the tradeoff. However, for light-limited applications such as fluorescence microscopy, this is a significant problem.

In order to address this problem, we have built a scanning system based on a microlens array. This is a small replicated optical element consisting of an array of microscopic lenslets, typically formed in a regular (square or hexagonal) grid. Lenslet diameters (and thus the pitch between elements) are on the order of tens to hundreds of microns. As in macroscopic convex lenses, if collimated light is passed through the lenslet, the light will be focused to a point at the focal length of the lens. With an array, most of the light is focused in a grid of illuminated points. Each of these points is a much better approximation to a Gaussian profile than is achieved with the LCD or DLP projection arrays. The illumination pattern can be scanned by mechanical translation of the microlens array, over

relatively short distances, comparable to the pitch of the lenslet array. Because exposures are made in parallel, shifts in the grid occur at low frequencies—typically 10–1000 Hz. The comparatively low speeds and small distances of translation allow a range of low-cost technologies to be used.

Figure 4 illustrates two methods for scanned illumination using such a lens array. The lenslets are illustrated by the larger circles in Figure 4; focused points of illumination are illustrated by the smaller circles. Figure 4A illustrates partial sampling of the image plane produced by translation of the microlens array in two dimensions. The illustrated grid is produced by three positions of the microlens array. By additional translations in two dimensions, the entire image plane can be sampled. Figure 4B illustrates a novel alternative scanning geometry achieved by tilting the microlens array. This configuration achieves high density sampling by translation along a single dimension. The most obvious strategies for scanning the array include rotary or linear stepping motor drives, galvanometer drives; or piezoelectric or ceramic devices. Mechanical scanning reintroduces concerns about system reliability, vibration, and the resolution and reproducibility of positioning. However, the performance requirements are comparable to modern mass-storage devices and thus we can use well-developed technical solutions. We have built a prototype scanner based on precision stepping motors, however galvanometer systems, or piezoceramic actuators may have advantages.

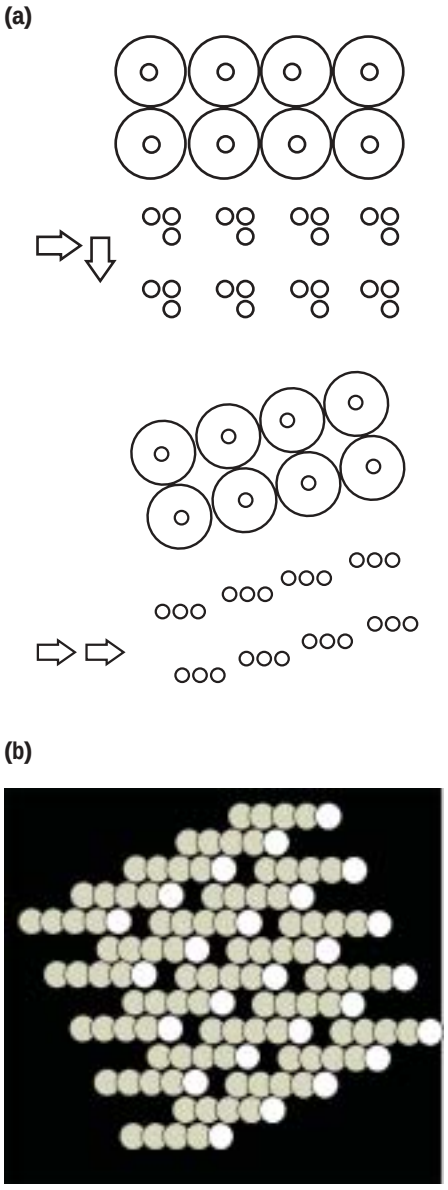
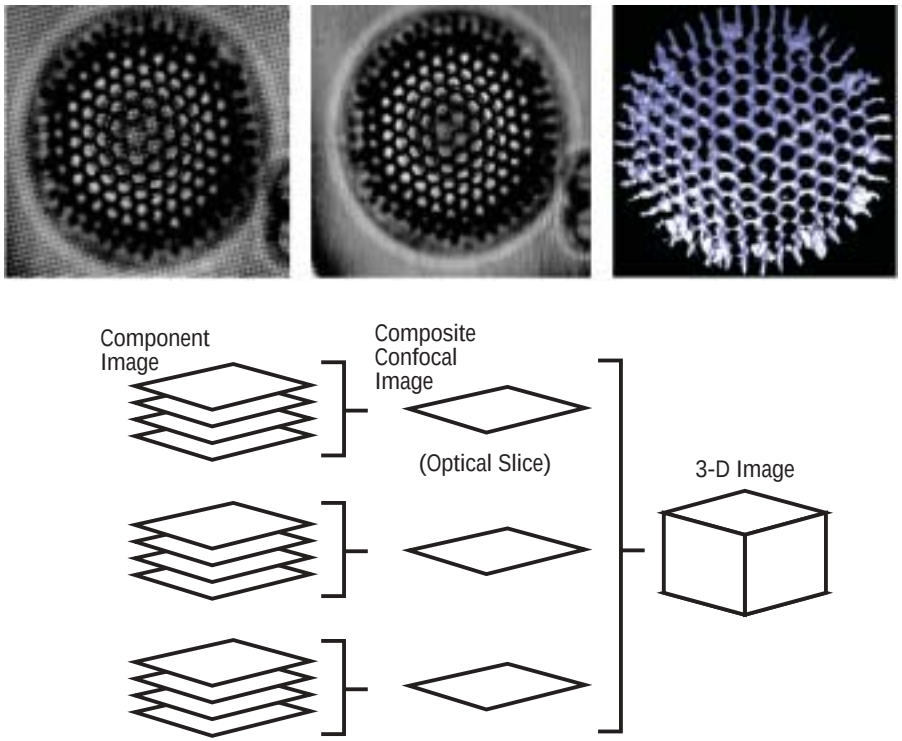


Figure4. Scanned illumination using a microlens array. (a) A 2-D raster scan using two actuators. (b) A 2-D raster scan using a single actuator.

The second key technical strategy is the use of a "virtual pinhole"—a synthetic aperture constituted after readout of a solid-state video imaging array—typically a CCD, charge-injection device (CID), photodiode-array camera, or similar device. In general, the synthetic aperture is implemented in software on the host computer or in a specialized digital signal-processing engine after digital image acquisition. These computations predict how much light would have passed through a physical aperture located in the focal plane of the microscope. We have demonstrated several workable algorithms, including some that are very efficient to compute. Because functional parameters are implemented in software, it is possible to adjust key parameters (such as effective aperture size) to optimize image tradeoffs, even after the basic data are acquired.

The system has a great deal of flexibility not available in other confocal systems. The detector can be operated as a conventional video camera, allowing easy focusing and survey of the microscope image field. The imager can be readily configured to operate in transmitted light modes as well as the reflected light or epifluorescence modes offered by most confocal microscopes. A number of image contrast mechanisms can be digitally synthesized; *e.g.*, transmitted light or dark field (scattered light) images can be produced by changing the calculation used to create the synthetic aperture. Multiple images can be computed from a single data set to optimize image data for a given application.

Our system provides confocal imaging capabilities to existing research microscopes, for little more than the cost of a conventional microscope with digital video capability. The system initially has been developed for confocal transmission imaging, because this is a commercially under-served application. Component systems are designed for flexibility; reflected light and fluorescence imaging capabilities use the same components. The imaging system provides a family of instruments, optimized for different applications: a spectrally-resolved microscopic imaging system that produces a complete spectrum for each image pixel; a high-performance system with extended sensitivity, speed, and dynamic range; and a confocal and spectroscopic endoscope system—rugged and light weight because no moving parts are required. We anticipate that other advanced systems based on these ideas will be developed in future work.



Confocal image reconstruction of a simple organism (diatom)

Figure5. Data Flow in the VPM: A series of component images are collected. Each is spatially subsampled using patterned illumination, produced by a MLA in this example. A composite confocal image (an optical slice) is reconstructed as a linear combination (a weighted sum) across the stack of component images. This operation implements the synthetic aperture. A 3-D image is reconstructed from a stack of confocal slices.

The principal application of existing confocal microscopes is in biological research studies of cell structure and function. Studying intact, functioning cells *in vivo*—for example, looking at how nerve cells process information in real time—could help researchers understand how the brain works or explore the root cause of crippling neuro-degenerative diseases. Our VPM technology promises to extend the technical advantages of confocal imaging to many applications in science and technology that depend on the flexibility and functionality of conventional video microscopy: biological research, histology and pathology studies, geological sample characterization, material science, and electronics manufacturing. Because the VPM system is compact, flexible, and free of alignment problems, it will facilitate the development of confocal endoscopes. The marriage of confocal and spectral imaging capabilities with endoscopy will provide new diagnostic methods that are minimally invasive in accessible areas.

Summary

By integrating state-of-the-art technologies in optics, electronics, and digital computing, we have developed a confocal imaging system for standard optical microscopes at much lower cost and with improved efficiency, reliability, and ease of use. Ours is a very flexible, high-performance imager, adaptable to a number of imaging configurations and applications not feasible or practical with other confocal imaging systems.

Our complete hardware system consists of imaging optics, typically supplied by a standard research microscope; a system for scanned illumination; a standard or high-performance solid-state video camera; and a computer system for image acquisition, scan control, and image reconstruction.

We have demonstrated several workable systems for electronically or electromechanically scanned illumination of many points in parallel. These methods have different strengths: LCD technology is flexible and compact; DLP technology is flexible and has high performance; MLA technology is compact and optically efficient. All methods provide adequate performance for at least some applications, at low cost. We have developed data-acquisition systems that control illumination scanning and perform image acquisition and processing. We have developed two classes of reconstruction method: an efficient calculation for real-time implementation, and advanced high-resolution algorithms that require substantial processing off-line. The combination of flexibility, high performance, and low cost in confocal imaging offered by our system should find a wide range of applications in science and technology.

References and Further Reading

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About the Authors

John George, Ph.D. is a Technical Staff Member in the Biophysics group (P-21) at Los Alamos National Laboratory. He came to Los Alamos as a Postdoctoral Fellow in the Life Sciences Division after completing his Ph.D. in Physiology from Vanderbilt University. His dissertation research, on the biochemical and ionic mechanisms of visual transduction in photoreceptor cells was conducted in the Laboratory of Chemical Physics at the National Institutes of Health. He has an educational background in biophysics, physiology, and computer science, and extensive experience in the development of biophysical instrumentation and electronic imaging. He is the inventor of the Virtual Pinhole Confocal Microscope and leads the continuing development of hardware and software systems and applications of the technology.

David Rector, Ph.D. is a Technical Staff Member in the Biophysics group (P-21) at Los Alamos National Laboratory. After completing his Ph.D. in Neuroscience at UCLA, he came to Los Alamos as a Director's Postdoctoral Fellow. He is an experimental neuroscientist with a background in digital computing as well as the design and development of high-performance electronic systems, in particular for physiological and image data acquisition. His work has demonstrated the feasibility of optical imaging of fast dynamic processes associated with the functional activation of neural tissue. Rector works on the illumination subsystem, and image-reconstruction algorithms and has primary responsibility for the development of the data acquisition and processing subsystem.